Journal of Surgical Oncology 61:42–48 (1996)

Cytosine Deaminase Gene as a Potential Tool for the Genetic Therapy of Colorectal Cancer

SIMON ROWLEY, MD, FRCS, MARCUS LINDAUER, MD, JOHANNES F. GEBERT, PhD, UWE HABERKORN, MD, FRANZ OBERDORFER, PhD, ULRICH MOEBIUS, PhD, CHRISTIAN HERFARTH, MD, AND HANS-KONRAD SCHACKERT, MD
From the Surgery Clinic, University of Heidelberg (S.R., M.L., J.F.G., C.H., H.-K.S.), Heidelberg, Germany; and the German Cancer Research Center (U.H., F.O., U.M.), Heidelberg, Germany

The bacterial enzyme cytosine deaminase (CD) catalyzes the conversion of 5-fluorocytosine (5-FC) to the lethal 5-fluorouracil (5-FU) and so provides a useful system for selective killing of gene-modified mammalian tumor cells. Cloning of the CD gene from *Escherichia coli* and expression in human tumor cell lines enabled these cells to convert ^{3}H-labeled 5-FC into ^{3}H-5-FU. Two CD-expressing human tumor cell lines (adenocarcinoma cell line KM12 and glioblastoma cell line T1115) became 200-fold more sensitive to 5-FC than the nonexpressing parental cell lines. At least 90% of the cells are killed within 7 days. CD-expressing cells are able to kill nonexpressing cells when grown in the same culture flask (bystander effect). The CD gene may be used as a suicide system for in situ chemotherapy or as a safety mechanism abrogating the expression of other genes.

KEY WORDS: **colorectal cancer, in situ gene therapy, cytosine deaminase**

INTRODUCTION

Colorectal cancer is the second most common form of cancer in North America and Europe, affecting about 150,000 people per year in the United States [1]. Surgery will cure approximately 50% of patients, whilst adjuvant therapy may prevent a further 10,000 deaths. Nevertheless, a significant number of patients still die from recurrent disease, with the liver being the most common site for metastases. Chemotherapy with 5-fluorouracil (5-FU) may be of some value in individual cases but can be limited by side effects when used systemically. Liver resection is only applicable to the minority of patients [2–5] and systemic chemotherapy of inoperable or recurrent disease is unlikely to have any impact on survival [6], although other forms of treatment such as immune therapy using a cytokine, recombinant interleukin-2 (IL-2) [7], are being explored.

With increasing interest in the application of gene therapy for cancer, a more locally restricted chemotherapeutic approach might improve results in the outcome of disease. Such locally restricted gene therapy might be feasible by transfer and expression of suicide genes at the site of the disease. Two nonmammalian enzymes, the herpes simplex virus thymidine kinase (HSVtk) and the bacterial cytosine deaminase (CD), have the potential for killing mammalian cells by converting nontoxic substrates, gancyclovir, and 5-fluorocytosine (5-FC) into the toxic compounds, gancyclovir phosphate and 5-FC [8]. The gene encoding CD has been cloned [9,10] and human cells have been shown to be capable of expressing this gene in vivo and in vitro [11–13]. There are, therefore, possibilities for the use of this gene as a tool for the destruction of transfected cells. Not only does this gene have therapeutic implications in itself but it may also be used as a control mechanism to abrogate the expression of other recombinant genes that have been transferred into human cells, such as IL-2.

As a first step towards the development of an in situ

Accepted for publication August 21, 1995.

Address reprint requests to Dr. Johannes F. Gebert, Chirurgische Universitatsklinik, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany.
Drs. Simon Rowley and Marcus Lindauer contributed equally to this work.

gene therapy of colorectal cancer we describe the cloning of the *Escherichia coli* (*E. coli*) gene encoding CD and its expression in a human colorectal cancer and a human glioblastoma cell line. We report a sensitive method of evaluation of CD enzymatic activity and demonstrate a bystander effect, although we show that cells containing this gene can recover once treatment with 5-FC is withdrawn. We also discuss the possibility of using this gene as a safety mechanism for controlling other genes, such as those expressing the human IL-2, which has been suggested as a possible method of genetic therapy for solid tumors.

MATERIALS AND METHODS

Cloning of the CD Gene

Two specific oligonucleotides which correspond to the published sequence of the CD gene (*cod A*) [9] were used for a polymerase chain reaction (PCR). The primer CDS1 (5′-GGG AAG CTT ACC ATG TCG AAT AAC GCT TTA C-3′) differed from the published sequence of the 5′ end of the CD gene at four positions (underlined). These 1) changed the GTG start codon to ATG, 2) more closely matched the Kozak consensus sequence [14], and 3) incorporated a *Hind III* restriction site. The second primer CDA2 (5′-GGG GGA TCC TCA ACG TTT GTA ATC GAT G-3′) corresponded to the 3′ end and contained a *Bam Hl* restriction site. A 1.3 kb DNA fragment of the modified CD gene was amplified by PCR using template DNA (1 μg) from a lactose utilizing *E. coli* K12 strain VE355 [15]. The PCR product was cloned into the *Hind III/Bam Hl* cut pBluescript vector pKS(−) creating pKS-CD and the DNA sequence of the modified CD gene was determined by dideoxy sequence [16].

Expression Vectors

The prokaryotic expression vector, plasmid pTrcHis-CD, was constructed by cloning a 1.3 kb *Xho I/Bam HI* fragment from pKS-CD into the *Xho I/Eco RI* cut vector pTrcHisA (Invitrogen, San Diego, CA) after the *Bam HI* site of the insert and *Eco RI* site of the plasmid were filled in with Klenow Fragment. Plasmid pTrcHisA-CD expresses a fusion protein that carries 6 histidine residues and 24 additional amino acids (enterokinase cleavage site) at its N-terminus. For CD expression in eukaryotic cells a 1.3 kb *Hind III/Xba I* fragment was isolated from pKS-CD and cloned into *Hind III/Xba I* cut pRc/RSV (Invitrogen), resulting in the plasmid pRSV-CD. This plasmid carried the respiratory syncytial virus (RSV) promotor/enhancer, a bovine growth hormone poly A sequence, and a neomycin resistance site, allowing for selection of G418-resistant cell lines. Plasmid pUHD10.1.IL2 contains a 550 bp fragment encoding human IL-2 cloned into the vector pUHD10.1 [17]. Plasmid pUHD10.1.IL2-CD expresses both CD and IL-2 and was constructed by blunt end cloning of a 2.2 kb *Bgl II/Pvu II* fragment of pRSV-CD into the *Xho I* site of pUHD10.1.IL2.

Transfection of Cell Lines

The human colorectal adenocarcinoma cell line KM12, subclone KM12L4 [18] (kindly provided by Dr. I.J. Fidler, MD Anderson Cancer Center, Houston, TX), and the glioblastoma cell line T1115 (kindly provided by H. Fischer, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were grown in basal Eagle medium (BME) with L-glutamine (Gibco/Bethesda Research Labs [BRL], Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; Serva, Heidelberg, Germany), penicillin, and streptomycin at 37°C with 5% CO_2. Cells were seeded at a density of 3×10^6 cells per 10 cm dish and transfected with a mixture of 10 μg linearized plasmid DNA and 20 μl Lipofectin (Gibco/BRL), in a total volume of 2 ml serum-free medium (Optimem, Gibco/BRL). After 6 hours incubation with intermittent shaking, the transfection mixture was removed and cells were grown for 48 hours in BME and G418 (500 μg/ml), and resistant clones were subsequently selected. Control cell lines contained only the RSV vector; this created the cell lines KM12.CD, KM12.RSV, T1115.RSV, and two T1115.CD clones (4 and 6). Stable integration of a plasmid pRSV-CD into the genomic DNA of the cell lines was confirmed by PCR and Southern blotting with hybridization to a 924 kb *Pst I/Nde I* internal fragment of the CD gene. Transient transfections were also performed under the same conditions as described for stable transfections.

CD Enzyme Assay

CD enzyme activity was determined both in the stably transfected cell lines and the bacterial strain, SØ 113, which lacks CD expression (a gift from Dr. S. Danielsen, Copenhagen, Denmark). SØ 113 and SØ 113 transfected with pTrcHisA-CD (SØ 113-pTrcHisA-CD) and the stable transfected human cell lines were grown to mid-log phase. 3×10^9 bacterial cells and 2.5×10^7 cells from human tumor cell lines were washed in phosphate-buffered saline and lyzed by sonification in lysis buffer (100 mM Tris-Cl, pH 7.4, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM dithiothreitol [DTT]). One hundred microliters of the resulting extracts were incubated with 100 mM Tris-Cl, pH 7.4, containing 10 μCi ^{3}H-5-FC, specific activity 203.5 GBq/mmol, radiochemical purity 99.7% (Moravek Biochemicals, Brea, USA). After incubation for 30 minutes (bacterial lysates) or 4 hours (human tumor cell lysates) at 37°C, total cellular protein was precipitated at 0°C with perchloric acid (0.6 M final concentration), the precipitate was pelleted at 20,000 *g* for 15 minutes at 0°C, and the supernatants neutralized with potassium hydroxide. Reversed-phase high-pressure liquid chromatography was performed as previously described [19]. A PRP 100 column (Eurogel, Knauer, 10

μ, 250 × 4.8 mm) was used with 8 mM NaH_2PO_4 buffer containing 1% TEA buffer at pH 2 and a flow rate of 1 ml/min. The ^{3}H-labeled 5-FU and 5-FC were detected using a Canberra A200 liquid scintillation flow detector (cell size 500 μl). Retention times were 2.8 minutes for 5-FC and 7.0 minutes for 5-FU.

Cell Killing, Recovery, and the Bystander Effect

Stably transfected KM12.CD cell lines were plated at 10,000 cells per well in BME containing G418 and varying concentrations of 5-FU and 5-FC (0.1, 0.5, and 50 mM). Cell numbers were determined immediately after seeding and 2, 4, 6, 8, and 10 days later using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay [20]. Having established cell killing of KM12.CD cells by 5-FC, experiments were performed to determine toxicity of 5-FC in the control cell line. Cells were exposed to different concentrations of 5-FC for 8 days and the viable cells determined by the MTT assay and compared to a control line grown without 5-FC. To judge if the cells could recover after treatment with 5-FC, the cells were incubated with 0.5 mM 5-FC in BME and treatment was terminated after 2, 4, or 8 days and the cells allowed to grow again and counted at regular intervals in a Neubauer chamber. As a control KM12.CD cells were grown untreated. The bystander effect was determined by mixing different concentrations of KM12.CD cells (100%, 50%, 30%, 10%, 5%) with nonexpressing cells (KM12.RSV), keeping the total cell numbers constant. These populations were grown in the presence of 0.5 mM 5-FC. Cell survival was measured with the MTT assay after 4, 8, and 10 days of treatment and compared to the number of cells in a pure KM12.RSV culture. To demonstrate the cell killing ability of pUHD10.1.IL2-CD, cells transiently transfected with the plasmid were treated with 0.5 mM 5-FC 24 hours after transfection and cell numbers were counted at regular intervals. IL-2 production was determined on the supernatant of transiently transfected KM12 cells by an IL-2-enzyme-linked immunosorbent assay (ELISA) test kit (H. Biermann, Bad Nauheim, Germany) at intervals up to 16 days. This was compared to the expression of cells transfected with pUHD10.1.IL2.

Sequencing of the CD gene showed there were two mutations in the sequence, one of which was at codon 69 which resulted in a change of amino acid from aspartate to histidine and a second mutation at codon 179 which did not result in amino acid change. The mutations did not alter the activity of the enzyme when compared to the wild type (data not shown).

Enzymatic Activity in Lysates From Bacteria and Human Tumor Cell Lines Expressing the CD Gene

The CD gene (*cod A*) has been amplified from *E. coli* DNA and cloned into eukaryotic and prokaryotic expression vectors. To verify the expression of enzymatically active CD encoded by these vectors, lysates from CD expressing bacteria (SØ 113-pTrcHisA-CD) and the transfected human colorectal and glioblastoma cell lines were analyzed by high-pressure liquid chromatography after addition of 5-FC. Formation of 5-FU could be demonstrated in CD-transfected cells compared to cell lines transfected with parental vectors alone (Table I). However, the level of activity varied significantly among the three transfected cell lines (KM12.CD and two different clones of T1115.CD, T1115.CD4 and T1115.CD6).

TABLE I. Enzymatic Activities of CD in Bacterial and Cellular Lysates*

	5-FU production (fM 5-FU/mg/min)
Bacteria	
SØ 113	1.6
SØ 113-CD	86
Cell lines	
KM12.RSV	0
KM12.CD	20
T1115.RSV	0
T1115.CD.4	84
T1115.CD.6	3.1

* Formation of ^{3}H-labeled 5-FU was determined by high-pressure liquid chromatography analysis. Numbers represent specific enzymatic activities (pmole ^{3}H-5-FU/min/mg protein) and were obtained from two independent experiments.

CD Expression in Human Tumor Cell Lines Mediates Sensitivity to 5-FC in Cell Culture

Survival of KM12.CD clones in the presence of different 5-FU and 5-FC concentrations was determined. 5-FU caused efficient cell killing in a concentration range 0.1–50 mM 5-FU within 4–6 days (Fig. 1a). 5-FC treatment also caused cell death in more than 90% of CD-expressing cells but required long exposure to the drug and even after 10 days never completely eradicated the population (Fig. 1b). Transfer and expression of the CD gene in these cells rendered them 200-fold more sensitive to 5-FC than the KM12.RSV control cells (Fig. 2). Similar experiments with the T1115.CD cells revealed that the T1115.CD4 cells were 27-fold more sensitive than the T1115.CD6 cells to 5-FC (data not shown), suggesting a correlation between CD activity (Table I) and cell killing. Overall these experiments indicate that the CD gene is capable of killing transfected cells.

Cell Recovery

Since 5-FC treatment, in contrast to 5-FU treatment, did not eradicate all CD-expressing cells in cell culture the potential for cell recovery after 5-FC treatment was determined (Fig. 3). Although there was a marked decline in cell numbers with 5-FC treatment, the surviving cells

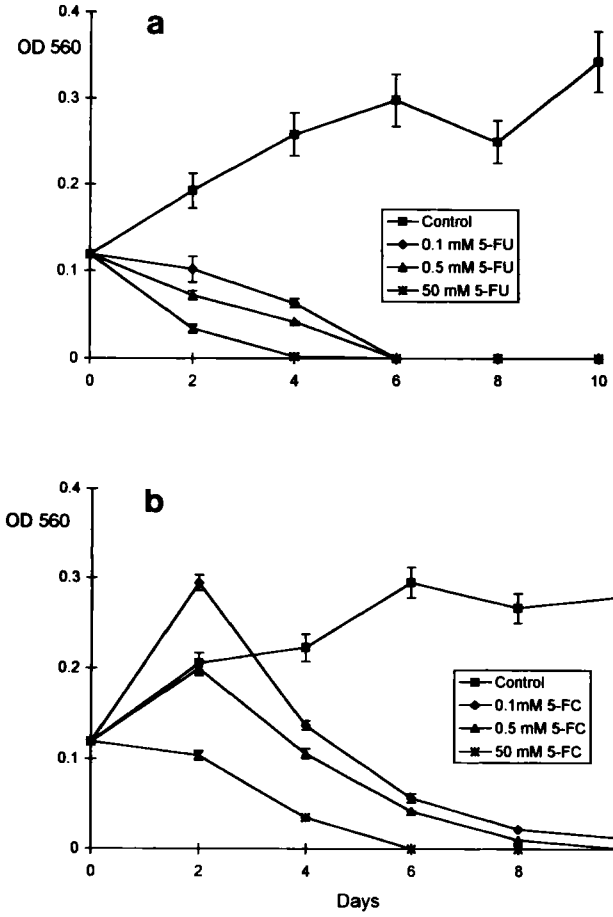

Fig. 1. **a:** Sensitivity of KM12.CD cells to different concentrations of 5-FU. Cells were grown in varying concentrations of the 5-FU and viable cells were determined by the MTT assay at two daily intervals. Absorbance at 560 nm indicates formation of formazan crystals and is directly proportional to the number of living cells. Each symbol represents values from three independent experiments and the SEMs shown by the error bars. **b:** Sensitivity of KM12.CD cells to different concentrations of 5-FC. Cells were grown in varying concentrations of the 5-FC and viable cells were determined by the MTT assay at two daily intervals.

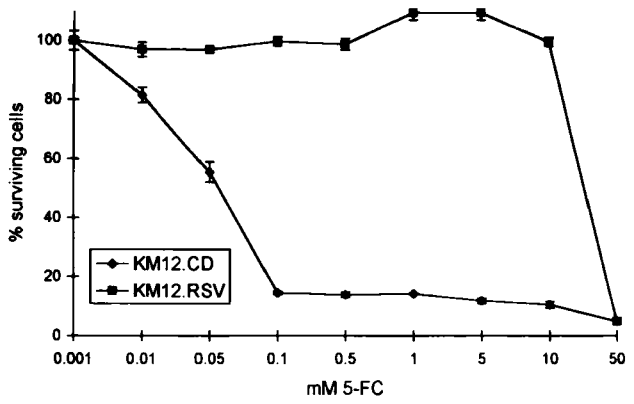

Fig. 2. Dose-dependent response experiment of KM12.CD and KM12.RSV cells. Cells were exposed to different 5-FC concentrations for 8 days and viable cells were determined by the MTT assay. The ordinate depicts the relative of viability in the absence of 5-FC compared to cells grown without 5-FC. The experiments were repeated in triplicate and the SEMs are shown.

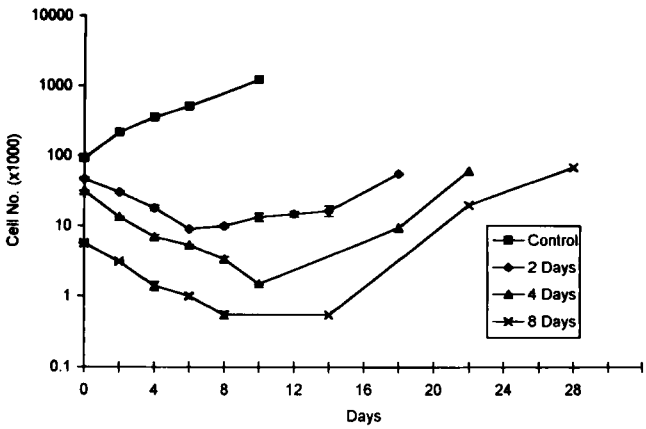

Fig. 3. Recovery of KM12.CD cells after 5-FC administration. KM12.CD cells were grown in the presence of 0.5 mM 5-FC for 2, 4, or 8 days. At day 0, 5-FC was removed and proliferation of surviving cells was monitored for up to 30 days. Cell numbers were obtained by counting viable cells in a Neubauer chamber. The results of three experiments are shown.

started growing once the treatment was removed and showed clear trends to full recovery.

Bystander Effect

The ability of CD-transfected cells to cause death of nontransfected, surrounding cells (the bystander effect) is shown (Fig. 4). When cell mixtures of 50% of KM12.CD/KM12.RSV cells were grown for 10 days in the presence of 5-FC, the majority of the cells were killed, including the cells which lack CD expression although this was less marked for lesser periods of time. This bystander effect was dependent on the time of the 5-FC treatment and the ratio of KM12.CD to KM12.RSV cells. Radioactive labeling of KM12.RSV cells with ^{3}H-thymidine confirmed these results.

Double Construct Activity

Cell killing activity by the double construct (pUHD10.1IL2-CD) was similar to transient transfection experiments using just the RSV-CD plasmid (Fig. 5), while IL-2 expression was almost identical to expression by pUHD10.1IL2 (data not shown), indicating that this double construct is capable of performing both of the functions for which it was designed.

DISCUSSION

Expression of the CD gene in eukaryotic cells has been previously reported in vivo and in vitro [10–12, 21], although there have been no previous descriptions of this gene being coexpressed with another therapeutically

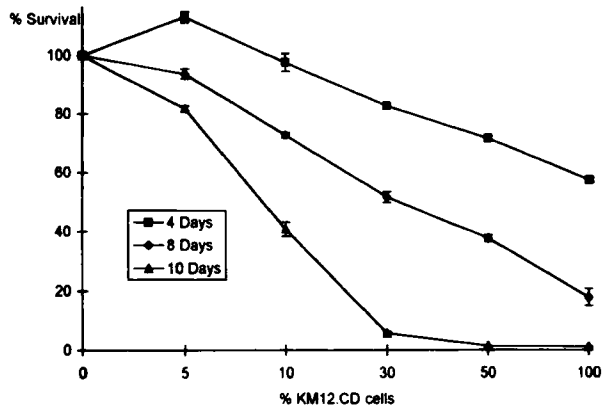

Fig. 4. KM12.CD cells confer a CD-mediated bystander effect. KM12.CD cells were cocultured with different ratios of KM12.RSV cells in the presence of 0.5 mM 5-FC. Cell numbers were determined at varying time periods and compared to the number of a pure KM12.RSV cell culture. The results were expressed as a percentage of this number (percentage survival).

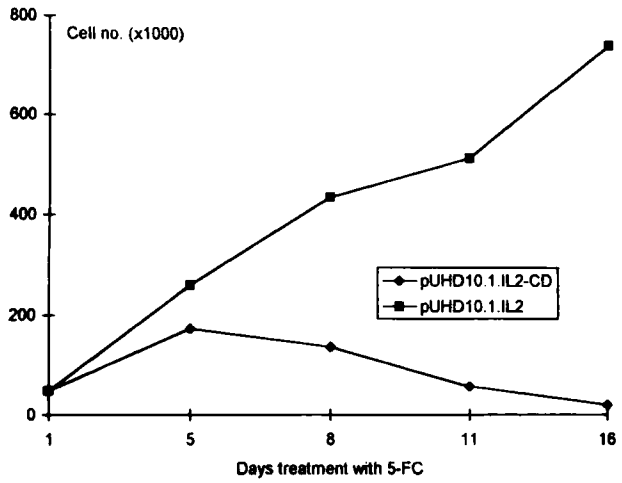

Fig. 5. Cell killing by pUHD10.1IL2-CD in a transient transfection. The cells were transient transfected and treated at 1 day with 5-FC at a concentration 0.5 mM and counted at regular intervals. There was marked inhibition of growth compared to the cells transfected with pUHD10.1.IL2 alone.

beneficial gene and the phenomenon of cell recovery has not been described. Since the ultimate goal is the use of the CD gene alone or in combination in clinical cancer treatment, the aim of these experiments was to characterize the gene and explore its potential in vitro before investigating its potential in vivo.

The cell killing experiments demonstrated that stable expression of CD can be achieved in human tumor cell lines and will lead to cell death when 5-FC is administered. Killing efficiency was similar in a concentration range from 0.1 to 50 mM 5-FC which corresponds to the concentration range recommended for therapeutic application [22]. This cell killing was achieved over a relatively long period of time, longer than 5-FU, although this could be due to different mechanisms for the uptake of 5-FC and 5-FU which would be consistent with the observation that cytosine entry into most cells proceeds via nonmediated permeation in contrast to the carrier-mediated transport of uracil [23]. Moreover, the enzymatic catalysis of 5-FC might be a slow reaction, never achieving the high endogenous 5-FU concentrations which are reached during 5-FU treatment. Such a slow formation of the 5-FU by the CD enzyme would not be surprising because it is known that the pyrimidine base, cytosine, is a 20-fold better substrate for CD than its fluorinated analog [24]. For a clinical application, time of treatment can be extended as appropriate to the action of the enzyme.

The results with two stably transfected clones of the glioblastoma cell line T1115 also might have implications for a clinical application of the CD suicide system. Different levels of CD activity correlated with different sensitivity towards 5-FC treatment. As a consequence, it may be assumed that increased expression or increased CD enzymatic activity could lead to improved killing efficiency. Focusing on the latter issue, CD mutants which metabolize the nontoxic drug 5-FC at the same rate or even better than the natural substrate, cytosine, would be useful. Studies addressing this are in progress.

We can confirm that the CD gene has a bystander effect, which was seen in vivo by one group [12] but not in vitro by another [11] and believe this should enhance its therapeutic benefits in a similar manner to the herpes simplex thymidine kinase gene which is currently under investigation in clinical trials [25,26]. In addition to 5-FU production in CD-expressing cells, the high-pressure liquid chromatography analyses also detected 5-FU production in the supernatant of the CD-expressing cell lines (U. Haberkorn, unpublished results). This would suggest two different levels of toxicity for the CD bystander effect: direct transfer of 5-FU via cell-to-cell contact and uptake of exogenous 5-FU from the extracellular environment. A factor which has not been reported before is the potential for cells to recover which was seen even after prolonged 5-FC administration. One explanation for this phenomenon could be that these cells were resistant to 5-FU, although our efforts to select spontaneous 5-FU-resistant mutants of KM12 in the presence of low 5-FU concentrations were unsuccessful. Alternatively, since only growing cells can be killed by CD-mediated 5-FU production, low 5-FU concentrations already produced by the majority of the cells might keep some cells locked in the G_0 phase of the cell cycle whilst the drug is present. Performing the same cell-killing experiments with CD-expressing cells arrested at different stages of the cell cycle will answer this question. This observation in cell culture is of considerable importance and is difficult to demonstrate in vivo using tumor xenografts. Further ex-

periments are underway to further delineate the mechanism of this.

Our preliminary experiments indicate that combined expression of the CD and IL-2 in KM12 cells can be achieved, although the rationale for IL-2 therapy alone in colorectal cancer is not clear. Since a mortality rate of 2% has been described for systemic IL-2 therapy [7,27], it has been suggested that IL-2 should be given locally as intracavitary, intrapleural, or intraarterial therapy [28,29]. This has met with some success and side effects have been noted to be considerably lower than equivalent systemic dosage; gene transfer may be the logical extension of this approach. There is also increasing evidence that combination of IL-2 with 5-FU may be beneficial in colorectal cancer. When using 5-FU in conjunction with IL-2 in advanced colorectal cancer, response rates of 10 and 29% have been reported [30,31], although again this has been associated with a significant toxicity when administered systemically [32]. In situ genetic therapy may provide the high local levels needed for a response whilst minimizing the systemic side effects. Notwithstanding the potential for in situ gene therapy, the addition of CD to IL-2-expressing plasmids may provide a measure of safety to the control of expression in ex vivo trials, such as those already underway [33].

In summary, our observations confirm that CD can be expressed and cause cell death in human colorectal cancer cells in vitro and confirm the reports of others of a bystander effect. It has, however, brought to attention the previously unreported phenomenon of the potential for cells to recover after treatment, a fact that we believe should be addressed when considering the potential usefulness of any adjuvant treatment of cancer treatment. The potential of CD as part of a doubly expressed construct has been demonstrated and it may have a role as a safety mechanism for other types of gene therapy.

ACKNOWLEDGMENTS

We thank Drs. H. Bujard and M. Gossen (Zentrum für Molekularbiologie Heidelberg, Heidelberg) for helpful discussions and the plasmid pUHD10.1, K. Haak (Chirurgische Universitätsklinik, Heidelberg) for providing plasmid pTrcHis-CD, Dr. W. Ansorge (European Molecular Biology Laboratory, Heidelberg) for technical advice on automated DNA sequencing, and Dr. S. Danielsen (University of Copenhagen) for providing the bacterial strain SØ 113. We gratefully acknowledge helpful suggestions on pyrimidine analysis from Dr. Keppler (Deutsches Krebsforschungszentrum, Heidelberg). S.R. was supported by the Alexander von Humboldt-Stiftung.

REFERENCES

1. Boring CC, Squires TS, Tong T: Cancer statistics 1993. CA 43:7–26, 1993.
2. Morrow CE, Grage TB, Sutherland DER, Najarian JS: Hepatic resection for secondary neoplasms. Surgery 92:610–614, 1982.
3. Holm A, Bradley E, Aldrete JS: Hepatic resection of metastases from colorectal carcinoma: Morbidity, mortality, and pattern of recurrence. Ann Surg 209:428–434, 1989.
4. Scheele J, Stangl R, Altendorf-Hofmann A: Hepatic metastases from colorectal carcinoma: Impact of surgical resection on the natural history. Br J Surg 77:1241–1246, 1990.
5. Doci R, Gennari L, Bignami P, et al.: One hundred patients with hepatic metastases from colorectal cancer treated by resection: Analysis of prognostic determinants. Br J Surg 78:797–801, 1991.
6. Cortesi E, Padovani A, Aloe A, et al.: Advanced colorectal cancer: Impact of chemotherapy on survival. J Surg Oncol (Suppl 2):112–115, 1991.
7. Rosenberg SA, Anderson WF, Blaese RM, et al.: Immunization of cancer patients using autologous cancer cells modified by the insertion of the gene for interleukin-2. Hum Gene Ther 3:75–90, 1992.
8. Moolten FL: Tumor chemosensitivity conferred by inserted Herpes Thymidine Kinase genes: paradigm for a prospective cancer control strategy. Cancer Res 46:5276–5281, 1986.
9. Danielsen S, Kilstrup M, Barilla K, et al.: Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. Mol Microbiol 6:1335–1344, 1992.
10. Austin EA, Huber BE: A first step in the development of gene therapy for colorectal carcinoma: Cloning, sequencing, and expressing of *Escherichia coli* cytosine deaminase. Mol Pharmacol 43:380–387, 1993.
11. Mullen CA, Kilstrup M, Blaese RM: Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: A negative selection system. Proc Natl Acad Sci USA 89:33–37, 1992.
12. Huber BE, Austin EA, Good SS, et al.: In vivo antitumour activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res 53:4619–4626, 1993.
13. Mullen CA, Coale MM, Lowe R, Blaese RM: Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. Cancer Res 54:1503–1506, 1994.
14. Kozak M: Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283–292, 1986.
15. Chaudhury AM, Smith GR: A new class of *Escherichia coli recBC* mutants: Implications for the role of RecBC enzyme in homologous recombination. Proc Natl Acad Sci USA 81:7850–7854, 1984.
16. Sanger F, Coulson AR: A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 94:441–448, 1975.
17. Deuschle U, Pepperkok R, Wang F, et al.: Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA and *lac* repressor. Proc Natl Acad Sci USA 86:5400–5404, 1989.
18. Morikawa K, Walker SM, Jessup JM, Fidler IJ: In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res 48:1943–1948, 1988.
19. Grün BR, Berger U, Oberdorfer F, et al.: Metabolism and actions of 2-deoxy-2-D-galactose in vivo. Eur J Biochem 190:11–19, 1990.
20. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63, 1983.
21. Huber BE, Austin EA, Richards CA, et al.: Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc Natl Acad Sci USA 91:8302–8306, 1994.
22. "The Pharmaceutical Codex, 11th ed." London: The Pharmaceutical Press, 1979.
23. Plagemann PGW, Woffendin C, Puziss MB, Wohlheuter RM: Purine and pyrimidine transport and permeation in human erythrocytes. Biochim Biophys Acta 905:17–29, 1987.
24. West TP, Shanley MS, O'Donovan GA: *Purification and some*

properties of cytosine deaminase from *Salmonella typhimurium*. Biochim Biophys Acta 719:251–258, 1982.
25. Culver KW, Ram Z, Wallbridge S, et al.: In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 256:1550–152, 1992.
26. Oldfield EH, Culver KW, Ram Z, et al.: A clinical protocol: Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous gancyclovir. Hum Gene Ther 4:39–69, 1993.
27. Lotze MT, Matory YL, Rayner AA, et al.: Clinical effects and toxicity of interleukin-2 in patients with cancer. Cancer 58:2764–2772, 1986.
28. Huland E, Huland H: Local continuous high dose interleukin-2: A new therapeutic model for the treatment of advanced bladder cancer. Cancer Res 49:1–6, 1989.
29. Eggermont AM, Goey SH, Bolhuis RL, Stoter G: Regional application of cytokine: Experience with various clinical protocols. Pharmacol Res 26:54–55, 1992.
30. Reid I, Sharpe I, Maxwell W, et al.: A phase 2 trial of recombinant interleukin-2 and 5-fluorouracil in patients with metastatic colorectal carcinoma. Eur J Surg Oncol 18:591–598, 1992.
31. Hamblin TJ, Sadhullah S, Williamson P, et al.: A phase-III study of recombinant interleukin 2 and 5-fluorouracil chemotherapy in patients with metastatic colorectal cancer. Br J Cancer 68:1186–1189, 1993.
32. Hiddeman W, Ruelfs C, Ottensmeier L, et al.: Interleukin-2 followed by fluorouracil and folinic acid in refractory colorectal cancer—Results of a clinical phase II study. Semin Oncol 19:225–227, 1992.
33. Rosenberg SA, Lotze MT, Muul LM, et al.: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high dose interleukin-2 alone. N Engl J Med 316:889–897, 1987.